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<b>(54) Title:</b> DETECTION OF MAMMARY TUMOR VIRUS-LIKE SEQUENCES IN HUMAN BREAST CANCER  <b>(57) Abstract</b>  The present invention relates to materials and methods for diagnosing breast cancer in humans. It is based, at least in part, on the discovery that a substantial percentage of human breast cancer tissue samples contained nucleic acid sequences corresponding to a portion of the mouse mammary tumor virus <u>env</u> gene. In contrast, such sequences were absent in almost all other human tissues tested.		

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## Description

### Detection Of Mammary Tumor Virus-Like Sequences In Human Breast Cancer

#### Cross-Reference to Related Application

This application is a continuation-in-part application of U.S. Serial No. 08/555,394, filed November 9, 1995.

#### Statement Regarding Federally Sponsored Research

5        This invention was made with funds from the U.S. government, which has certain rights in the invention.

#### Introduction

10        The present invention relates to materials and methods for diagnosing breast cancer in humans. It is based, at least in part, on the discovery that a substantial percentage of human breast cancer tissue samples contained nucleic acid sequences corresponding to a portion of the mouse mammary tumor virus env gene. In contrast, such sequences were absent in almost all  
15        other human tissues tested.

#### Background of the Invention

20        A large body of information has accumulated about the molecular biology of MMTV (reviewed in Slagle, B.L. et al., 1987, in "Cellular and Molecular Biology of Mammary Cancer", Kidwell et al., eds., Plenum Press, NY. pp 275-306). Mouse mammary tumor virus (MMTV) is associated with a high incidence of breast cancer in certain strains of mice (over 90% among females), and has been regarded as a potential model for human  
25        disease.

      The MMTV virus does not carry a transforming oncogene, but rather acts as an insertional mutagen with several proviral insertion loci designated int-1

or wnt-1 (Nusse R. et al., 1982, Cell 31:99-109) int-2  
(Peters, G. et al., 1983, Cell 33:369-377) int-3  
(Gallahan, D. et al., 1987, J. Virol. 61:218-220) int-4  
(Roelink, H. et al., 1990, Proc. Natl. acad. Sci. USA  
5 87:4519-4523) and int-5 (Morris, V.L., et al. 1991,  
Oncogene Research 6:53-63), which encode for growth  
factors or other related proteins. These genes are not  
expressed in normal mammary tissue but become activated  
after integration of MMTV provirus into the adjacent  
10 chromosomal DNA.

The human homolog of the int-2 locus has been  
located on chromosome 11 (Casey, G. et al., 1986,  
Mol. Cell Biol. 6:502-510) and has been found amplified  
(in 15% of the breast cancers) and also expressed  
15 (Lidereau, R. et al., 1988, Oncogene Res 2:285-291;  
Zhou, D.J. et al., 1988, Oncogene 2:279-282; Liscia,  
D.S. et al., 1989, Oncogene 4:1219-1224; Meyers, S.L.  
et al., 1990, Cancer Res 50:5911-5918). It may be  
significant that in tumors from Parsi women, who have a  
20 high incidence of breast tumors, the int-2 locus is  
amplified in 50% of the cases (Barnabas-Sohi, N. et  
al., 1993, Breast Dis. 6:13-26). The amplification of  
int-2 and other genes in 11q13 is indicative of poor  
prognosis (Schuwring, E. et al., 1992, Cancer Research  
25 52:5229-5234; Champeme, M-H, et al., 1995, Genes,  
Chromosomes and Cancer 12:128-133). Both mouse and  
human int-2 have been sequenced (Moore, R. et al.,  
1986, EMBO J 5:919-924). The gene encodes a protein of  
about 27 kilodaltons (KD) which shows homology to both  
30 basic and acidic fibroblast growth factors (Dickson, C.  
et al. 1987, Nature (London) 326:833).

However, efforts to demonstrate the presence of  
viruses in human breast cancer through search for viral  
particles, immunological cross-reactivity, or sequence  
35 homology have yielded contradictory results. Detect-  
able MMTV env gene-related antigenic reactivity  
has been found in tissue sections of breast cancer

(Mesa-Tejada et al., 1978, Proc. Natl. Acad. Sci. USA 75:1529-1533; Levine, P. et al., 1980, Proc. Am. Assoc. Cancer Res. 21:170; Lloyd, R. et al., 1983, Cancer 51:654-661), breast cancer cells in culture (Litvinov, S.V. and Golovkina, T.V., 1989, Acta Virologica 33:137-142), human milk (Zotter S. et al., 1980, Eur. J. Cancer 16:455-467) in sera of patients (Day, N.K. et al., 1981, Proc. Natl. Acad. Sci. USA 78:2483-2487), in cyst fluid (Witkin, S.S. et al., 1981, J. Clin. Invest. 67:216-222) and in particles produced by a human breast carcinoma cell line (Keydar, I. et al., 1984, Proc. Natl. Acad. Sci. USA 81:4188-4192). Sequence homology to MMTV has been found in human DNA under low stringency conditions of hybridization (Callahan, R. et al., 1982, Proc. Natl. Acad. Sci. USA 79:5503-5507) and RNA related to MMTV has been detected in human breast cancer cells (Axel, R. et al., 1972, Nature 235:32-36). The presence of MMTV related sequences in lymphocytes from patients with breast cancer has been reported (Crepin, M. et al., 1984, Biochem. Biophys. Res. Comm. 118:324-331), as well as detection of reverse transcriptase (RT) activity in their monocytes (Al-Sumidaie, A.M. et al., 1988, Lancet 1:5-8). May and Westley (May and Westley, 1989, Cancer Research 49:3879-3883) have reported the presence of MMTV-like sequences arranged as tandem repeats only in DNA from breast cancer cells.

These results have been difficult to interpret, and theories linking MMTV or a related virus with human breast cancer have fallen out of favor, in view of the relatively recent discovery of human endogenous retroviral sequences ("HERs"; Westley, B. et al., 1986, J. Virol. 60:743-749; Ono, M. et al., 1986, J. Virol. 60:589-598; Faff, O. et al., 1992, J. Gen. Virology 73:1087-1097). Data which could be interpreted to demonstrate the presence of MMTV-related sequences could be more readily explained by endogenous human

retroviral sequences. Adding further confusion to the picture, env-gene related antigenicity has been detected in epitopes of human proteins (Hareuveni, M. et al., 1990, Int. J. Cancer 46:1134-1135).

5    Brief Summary of the Invention

          The present invention relates to methods for diagnosing breast cancer in humans in which the presence of mouse mammary tumor virus env gene-like sequences bears a positive correlation to the existence of malignant breast disease. It is based, at least in part, on the discovery that 38 to 40 percent of human breast cancer tissue samples tested contained gene sequences homologous to the mouse mammary tumor virus env gene that are substantially absent from other human tumors and tissues. The invention also relates to methods for diagnosing breast cancer in humans in which the presence of retrovirus proviral fragments substantially homologous to the env gene and/or 3' LTR sequence of MMTV are detected. The molecular probes used in these experiments were designed to avoid cross-hybridization with endogenous human retroviral sequences. The present invention further provides for compositions of molecular probes which may be utilized in such diagnostic methods.

25   Brief Description of the Figures

FIGURE 1: Amplification of 660 bp of MMTV-like env gene. DNA was extracted from frozen tissues. PCR was performed using primers 1 and 3. A: 2% agarose gel electrophoresis. B: Southern blot hybridization using 5'<sup>32</sup>P-end-labeled probe 2. Lanes 1 and 3: breast cancer; lanes 2 and 4: normal breast; lane 5: control reaction (no DNA); lane E: MMTV env gene. M: molecular weight marker. Arrow indicates 510 bp band.

FIGURE 2: Nested PCR. A: 2% agarose gel electrophoresis. 1: Amplification of 686 bp of MMTV-like env

gene sequences using primers 1 and 4 and the product of reaction A 1 as template. 2: Amplification of 250 bp of MMTV-like env gene sequences using primers 2 and 3. B, 1 and 2: Southern blot hybridization of the amplified products using probe 5'-<sup>32</sup>P end-labeled probe 2a.

**FIGURE 3:** Amplification of 250 bp of MMTV-like env gene. DNA was extracted from paraffin-embedded tissue sections. PCR was performed using primers 2 and 3. A: 2% agarose gel electrophoresis. B: Southern blot hybridization using 5'-<sup>32</sup>P-labeled probe 2a. Lane 1: normal breast; lanes 2 to 5: breast cancer; lane E: MMTV env gene. M: molecular weight marker. Arrow indicates 298 bp band.

**FIGURE 4:** Nucleotide sequence of the cloned MMTV env gene-like sequences as compared to the env sequences of the GR and BR6 strains of MMTV using the GCG program. \*:potential glycosylation site, |:mismatch to MMTV.

**FIGURE 5:** Southern blot hybridization of genomic DNA. DNA was extracted from frozen tissues or cell lines, digested with EcoRI and transferred to nitrocellulose paper. Hybridization with <sup>32</sup>P-labeled clone 166. DNA from A, B, and G: env gene positive breast cancer; C and D: env negative breast cancer; E and F: normal breast; H: MCF-7 cells. M: molecular weight marker, Arrow indicates 9kb band.

**FIGURE 6:** Southern blot hybridization of genomic DNA. Experimental conditions as in Fig. 5. DNA from A and B: env negative breast cancer; C and D: env positive breast cancer; E: molecular weight marker (non-labelled); F. to H: normal breast. Arrow indicates position of 9 kb marker.

**FIGURE 7:** Map of MMTV.

**FIGURE 8:** Comparison of the nucleic acid sequence of mouse mammary tumor env gene ("MMTENV"), showing residues 976-1640, with the nucleic acid sequence of a

representative 660 bp sequence obtained by PCR reaction of DNA from human breast cancer tissue ("MS1627").

FIGURE 9: Sequence of an about 2.6 kb MMTV-like fragment detected in a human breast carcinoma.

## 5 Detailed Description of the Invention

The present invention relates to methods and compositions for diagnosing breast cancer in humans.

The present invention provides for compositions comprising an isolated and purified nucleic acid  
10 molecule which (i) hybridizes to a gene of mouse mammary tumor virus; (ii) is present in at least 20 percent of DNA samples prepared from breast cancer tissue of different human subjects; and (iii) is present in less than 5 percent of DNA samples prepared  
15 from tissues other than breast cancer tissue from different human subjects. A "gene of mouse mammary tumor virus" includes, but is not limited to, the gag, pol, and env genes and the 5' LTR and 3' LTR sequences of MMTV. In preferred embodiments of the invention,  
20 the mouse mammary tumor virus (hereafter "MMTV") gene is the env gene and/or the 3' LTR sequence. The term "hybridize" is used to refer to routine DNA-DNA or DNA-RNA hybridization techniques under what would be regarded, by the skilled artisan, as stringent  
25 hybridization conditions. The phrase "is present" indicates that a native form of the molecule, in an unpurified state (for example, as part of chromosomal DNA), may be detected by a standard laboratory technique, such as Southern blot or polymerase chain  
30 reaction (PCR). To be "present", the molecule may be detectable by one technique but not others. To be present in "less than 5 percent of DNA samples prepared from tissues other than breast cancer tissue from different human subjects", all non-breast cancer tissue  
35 samples are considered together, but the total number of samples must be large enough to give the 5 percent



value statistical significance that would be reasonable to the skilled artisan.

In order to identify such a nucleic acid molecule, the sequence of MMTV may be compared, using a computer database, to known human DNA sequences, and portions of MMTV which are less than or equal to 25 percent homologous to a human sequence may be selected for further study. The term "homologous", as used herein, refers to the presence of identical residues; for example, a first sequence is considered 25 percent homologous to a second sequence if it shares 25 percent of the residues of the first sequence. Since there is relatively greater likelihood that MMTV may bear similarity to human retroviral-like sequences, it may be preferable to evaluate whether a particular MMTV nucleic acid sequence is homologous to such sequences, for example, as endogenous human retrovirus sequences. A prototype of such viruses is HERV-K10 (Ono, M. et al., 1986, J. Virol. 60:589-598).

Once an MMTV gene sequence which is less than or equal to 25 percent homologous to a human DNA sequence, such as a human endogenous retroviral sequence, is identified, the presence of nucleic acid molecules having the MMTV gene sequence in human breast cancer tissues and other tissues may be evaluated. Such evaluations may be performed either by Southern blot techniques, or, preferably, by polymerase chain reaction (PCR) techniques, which are more sensitive. In such a way, MMTV gene sequences which (i) hybridize to at least 20 percent of DNA samples prepared from breast cancer tissue of different human subjects and (ii) hybridize to less than 5 percent of DNA samples prepared from human tissues other than breast cancer tissues may be identified. A nucleic acid molecule having a MMTV gene sequence which satisfies these requirements may then be used in diagnostic methods which detect the presence of such sequence in human

breast tissue by standard techniques, including PCR techniques which assay for the presence of the molecule, but also, where appropriate, Southern blot, Northern blot, or Western blot techniques, to name but  
5 a few.

In preferred embodiments, the present invention relates to a portion of MMTV localized between MMTV env gene sequences 976 and 1640 (Majors, I.E. and Varmus, H.E., 1983, J. Virol. 47:495-504; see Fig. 7). This  
10 about 660 bp sequence (hereafter, "the 660 bp sequence") has been found to exhibit low (16 percent) homology to the prototype human endogenous retrovirus HERV-K10, using the IBI/Pustell Sequence Analysis Program, and has also been shown to be present in 121  
15 (38.5%) of 314 unselected breast cancer tissue samples, in cultured breast cancer cells, in 2 of 29 breast fibroadenomas (6.9%) and in 2 of 107 breast specimens from reduction mammoplasties (1.8%). The sequence was not found in normal tissues including breast, lympho-  
20 cytes from breast cancer patients nor in other human cancers or cell lines (see example section, infra). Similarly, an about 250 bp sequence (hereafter "the 250 bp sequence"), between positions 1388 and 1640 in the env gene, and therefore falling within the 660 bp  
25 sequence, was detected in 60 (39.7%) of 151 breast cancer, and in one of 27 normal breast samples assayed from paraffin-embedded sections. Cloning and sequencing of the 660 bp and 250 bp sequences demonstrated that they are 95-99% homologous to MMTV env gene, but  
30 not to the known human endogenous retroviruses ("HERs") nor to other viral or human genes (<18%).

In another preferred embodiment, the present invention relates to a nucleic acid molecule which corresponds to a retroviral genomic fragment which has  
35 substantial homology to 3' LTR and/or env gene of the MMTV genome, and is found in a substantial percentage of breast cancer samples. By substantial percentage is

meant at least 20% of tested breast cancer samples. Such a sequence is preferably comprised of the 3' LTR region and all or part of the env gene, although it may include more sequences of a retroviral genome. Most  
5 preferably, the sequence is at least comprised of an about 2.6 kb fragment which comprises the 1,228 base pair (bp) sequence of the 3' LTR sequence and 1,336 bp of the env gene sequence of MMTV (Fig. 9) (SEQ ID NO:20). When compared with the two strains of MMTV C3H  
10 and BR6, the sequence homology was 90.8% and 90.7%, respectively. When compared with the endogenous retroviral sequences (HUMERKA), sequence homology was only 58% in 36 bp and 71% in 74 bp.

Retrovirus proviral sequences can be detected by  
15 PCR technology using primers derived from the MMTV genome. Such primers include primer 5L, containing the nucleotides 7376-7395 of the MMTV BR6 genome (5'-3': CCAGATCGCCTTTAAGAAGG) (SEQ ID NO:11) and primer LTR3, containing nucleotides 9918-9927 of the MMTV BR6 genome  
20 (5'-3': CGAACAGACACAAAGCGACG) (SEQ ID NO:19). Other primers which correspond to or are homologous to MMTV sequences can be used as primers. Nucleotide fragments which correspond to or are homologous to the retroviral sequences isolated from the breast cancer samples can  
25 also be used to amplify additional retroviral fragments from the samples. Long PCR techniques can be used to amplify longer stretches of a proviral sequence.

The present invention provides for compositions comprising an isolated and purified nucleic acid  
30 molecule which hybridizes to the about 2.6 kb retroviral fragment shown in Fig. 9 under stringent conditions or is at least 90 percent homologous to said fragment using the MacVector homology determining program which may be used to diagnose breast cancer in  
35 a subject, using methods which include PCR and Southern blot methods.

Nucleic acids having the 660 bp sequence, the 250 bp sequence, or all or part of the about 2.6 kb sequence, may therefore be used, according to the invention, to diagnose breast cancer in a subject, using methods which include PCR and Southern blot methods. Where PCR methods are used, primers such as those listed in Table 1, below, may be utilized.

The present invention provides for compositions comprising essentially purified and isolated nucleic acid having the 660 bp sequence or the 250 bp sequence or an at least five bp, and preferably greater than or equal to ten bp, subsequence thereof. In order to maintain the desired specificity, such nucleic acid molecules may preferably contain sequence falling within the 660 bp sequence, but preferably do not contain sequences from other portions of the MMTV genome, which may, undesirably, hybridize to human sequences which are not breast cancer specific, such as HERs. Accordingly, the present invention provides for compositions wherein the isolated and purified nucleic acid molecule comprises at least a portion having a nucleic acid sequence which hybridizes to a region of the mouse mammary tumor virus env gene between residues 976 and 1640, or between residues 1388 and 1640, and wherein the isolated and purified nucleic acid molecule does not hybridize to any other region of the MMTV genome.

The 660 bp sequence, in various embodiments, may have a number of nucleotide sequences. For example, in one embodiment, the 660 bp sequence may have a sequence as set forth in Fig. 8 and designated "MMTENV-like sequence" (SEQ ID NO:17), which depicts the MMTV env sequence between residues 976 and 1640. In a second series of embodiments, the 660 bp sequence may have a sequence as set forth in Fig. 8 and designated "MS1627" (SEQ ID NO:18), which depicts a predominant sequence for the 660 bp sequence as it has been defined by

sequencing analysis of the products of PCR reactions using DNA from human breast cancer tissues. In still further embodiments, the 660 bp sequence may have various other nucleotide sequences obtained by  
5 sequencing the results of PCR reactions to detect the presence of 660 bp sequence in human breast cancer tissues.

In related embodiments, the present invention provides for compositions comprising PCR primers  
10 that may be used to detect the presence of the forementioned molecules or other MMTV-like sequences. For example, the compositions may comprise one or more of the following primer molecules (5' - 3'):  
CCTCACTGCCAGATC (SEQ ID NO:1); GGGAATTCCTCACTGCCAGATC  
15 (SEQ ID NO:2); CCTCACTGCCAGATCGCCT (SEQ ID NO:3);  
TACATCTGCCTGTGTTAC (SEQ ID NO:4); CCTACATCTGCCTGTGTTAC  
(SEQ ID NO:5); CCGCCATACGTGCTG (SEQ ID NO:6);  
ATCTGTGGCATACT (SEQ ID NO:7); GGGAATTCATCTGTGGCATACT  
(SEQ ID NO:8); ATCTGTGGCATACTAAAGG (SEQ ID NO:9);  
20 GAATCGCTTGGCTCG (SEQ ID NO:10); CCAGATCGCCTTTAAGAAGG  
(SEQ ID NO:11); TACAGGTAGCAGCACGTATG (SEQ ID NO:12);  
CGAACAGACACAAACACACG (SEQ ID NO:19).

The use of such compositions and molecules in PCR and Southern blot techniques is illustrated in the non-  
25 limiting examples set forth below. The correlation between the presence of the MMTV-related nucleic acid molecules described above and breast cancer allows such molecules and compositions to be utilized in the diagnosis of breast cancer. Accordingly, the present  
30 invention provides for a method of diagnosing breast cancer, wherein the detection of such nucleic acid molecules bears a positive correlation to the existence of breast cancer in a human. The results of such evaluation, together with additional clinical symptoms,  
35 signs, and laboratory test values, may be used to formulate the complete diagnosis of the patient.

In further related embodiments, the present invention provides for an essentially purified peptide encoded by a nucleic acid molecule which (i) hybridizes to a gene of MMTV; (ii) is present in at least  
5 20 percent of DNA samples prepared from breast cancer tissue of different human subjects; and (iii) is present in less than 5 percent of DNA samples prepared from tissues other than breast cancer tissue from different human subjects. In preferred embodiments, the  
10 MMTV gene is the env gene.

Such peptides may be used in the diagnosis of breast cancer. Accordingly, the present invention provides for a method of diagnosing breast cancer in a human subject, comprising detecting the presence of  
15 a peptide encoded by a nucleic acid molecule which (i) hybridizes to the env gene of a mouse mammary tumor virus; (ii) is present in at least 20 percent of DNA samples prepared from breast cancer tissue of different human subjects; and (iii) is present in less than  
20 5 percent of DNA samples prepared from tissues other than breast cancer tissue from different human subjects.

The present invention also provides for antibodies (including monoclonal and polyclonal) antibodies which  
25 specifically bind to such peptides. Such antibodies may be used in methods of diagnosing breast cancer, for example, but not by way of limitation, by Western blot, immunofluorescent techniques, and so forth.

In nonlimiting embodiments of the invention, the  
30 skilled artisan may evaluate MMTV-like nucleic acid molecules for regions which would be considered likely to encode immunogenic peptides (using, for example, hydropathy plots). Such peptides may then be sequenced and used to produce antibodies that may be employed in  
35 diagnostic methods as set forth above.

For example, certain peptides encoded by portions of the 660 bp sequence have been synthesized. These

peptides, which have the sequences LKRPGFQEHMI (SEQ ID NO:13) and GLPHLIDIEKRG (SEQ ID NO:14), have been used to produce antibodies in rabbits, and the resulting antisera have successfully identified breast cancer cells positive for MMTV env-like sequences by PCR assay. Other peptides encoded by 660 bp sequence which may be useful according to the invention include TNCLDSSAYDTA (SEQ ID NO:15) and DIGDEPWFDD (SEQ ID NO:16).

10           6.     Example: The Detection of Mouse Mammary Tumor Virus Env Gene-Like Sequences in Human Breast Cancer Cells and Tissues

                  6.1. Materials and Methods

                  DNA from breast cancer tissue and other human  
15   cancer tissues, human placentas, normal human tissues including breast, and from several human cell lines (including eight breast cancer cell lines), and two normal breast cell lines was extracted following the procedure of Delli Bovi et al. (1986, Cancer Res.  
20   46:6333-6338). The DNA was resuspended in a solution containing 0.05 M Tris HCl buffer, pH 7.8, and 0.1 mM EDTA, and the amount of DNA recovered was determined by microfluorometry using Hoechst 33258 dye (Cesarone, C. et al., 1979, Anal Biochem 100:188-197). Plasmids  
25   containing the cloned genes of MMTV were obtained from the ATCC, propagated in Escherichia coli cultures and purified using anion-exchange minicolumns (Qiagen) or by precipitation with polyethylene glycol (Sambrook J., et al., 1989, in "Molecular Cloning/A Laboratory  
30   Manual", Cold Spring Harbor). Oligonucleotide primers were synthesized at the core facilities of the Brookdale Molecular Biology Center at Mount Sinai School of Medicine.

                  Polymerase chain reaction (PCR) was performed  
35   using Taq polymerase following the conditions recommended by the manufacturer (Perkin Elmer Cetus)

with regard to buffer,  $Mg^{2+}$  and nucleotide concentrations. Thermocycling was performed in a DNA cycler by denaturation at 94° C for 3 min. followed by either 35 or 50 cycles of 94°C for 1.5 min., 50° C for 2 min. and 72°C for 3 min. The ability of the PCR to amplify the selected regions of the MMTV env gene was tested by using as positive templates the cloned MMTV env gene and the genomic DNA of the MCF-7 cell line, since it was shown to express gp52 immunological determinants (Yang, N.S., et al., 1975, J. Natl. Cancer Inst. 61:1205-1208). Optimal  $Mg^{2+}$ , primer concentrations and requirements for the different cycling temperatures were determined with these templates. The master mix as recommended by the manufacturer was used. To detect possible contamination of the master mix components, a reaction without template was routinely tested.  $\gamma$  DNA and control primers provided by the manufacturer were used as control for polymerase activity. As an internal control, amplification of a 120 bp sequence estrogen receptor gene was assayed using primers designed and generously provided by Dr. Beth Schachter, (Mount Sinai School of Medicine, N.Y.). In addition, primers for actin 5 gene amplification were also used.

The product of the PCR was analyzed by electrophoresis in a 2% agarose gel. A 1 kb DNA ladder (Gibco BRL) was used to identify the size of the PCR product. To determine if the amplified sequences of the middle region of the 660 bp faithfully reproduced the sequences of the env gene of MMTV, an 18-mer sequence within the env gene was used as a probe for the 660 bp amplified sequence. The 18-mer probe was 5' end-labeled with  $^{32}P$ -ATP using T4 polynucleotide kinase and purified by the NENSORB nucleic acid purification cartridge (NEN). Southern blot hybridization was performed using the conditions described by (Saiki et al., 1985, Science 230:1350-1354).



The product of the PCR (660 bp or 250 bp) was cloned directly from the reaction mixture into the TA cloning vector (Invitrogen) using the TA cloning kit and following the conditions recommended by the supplier. Direct cloning of the fragment isolated from the gel, was also performed. Plasmid DNA was purified by CsCl density gradient centrifugation or by precipitation with polyethylene glycol (Sambrook et al., 1989, in "Molecular Cloning/A Laboratory Manual", Cold Spring Harbor), restricted with HindIII and EcoRI, electrophoresed in 2% agarose gels and transferred to nitrocellulose filters. Southern blot hybridization was carried out using a 5'-terminal labeled internal probe as described above. Cloning procedures were performed in laboratories totally separate from those where PCR was carried out. Automated DNA sequencing (using Applied Technology Sequencer Model 373A) was performed in the Brookdale Molecular Biology Center. Sequence homology was determined using the IBI MacVector GenBank and GCG Programs.

To prevent contamination of the samples, processing of human tissues was performed in a laminar flow hood. DNA extractions were done in a chemical hood located in a different room from that where PCR was performed. PCR assays were assembled in a biological hood provided with ultraviolet light. Aerosol resistant tips and dedicated positive-displacement pipettes were used throughout. All equipment used for PCR (microcentrifuge, electrophoresis apparatus, pipettors) was cleaned each time with 10% sodium hypochlorite to assure DNA decontamination (Prince and Andrus, 1992, Biotechniques 12:358-36). After the initial experiments were performed, the plasmid containing the MMTV env gene was frozen and never used again, to avoid contamination. However, to detect plasmid contamination from our own env gene clones,

primers were designed to amplify plasmid sequences. All the authentic MMTV env positive samples were then tested and found negative for plasmid contamination.

Southern blotting and hybridization were performed as described (Southern, E.M., 1975, J. Mol. Biol. 98:503-517), using the 660 bp cloned sequences labeled by the random primer procedure (Feinberg, A.P., et al., 1983, Anal. Biochem. 132:6-13). Prehybridization and hybridization were performed in a solution containing 6 x SSPE, 5% Denhardt's, 0.5% SDS, 50% formamide, 100 µg/ml denaturated salmon testis DNA, incubated for 18 hrs at 42°C, followed by washings with 2 x SSC and 0.5% SDS at room temperature and at 37°C and finally in 0.1 x SSC with 0.5% SDS at 68°C for 30 min (Sambrook et al., 1989, in "Molecular Cloning/A Laboratory Manual", Cold Spring Harbor). For paraffin-embedded tissue sections the conditions described by Wright and Manos (1990, in "PCR Protocols", Innis et al., eds., Academic Press, pp. 153-158) were followed using primers designed to detect a 250 bp sequence.

## 6.2. Results

### 6.2.1. Selection of Specific MMTV Env Gene Sequences

A computer search for MMTV env gene homologous sequences was first performed, since sequence homology between the human endogenous retroviral sequences and MMTV had been described. The prototype of this group of human endogenous retroviruses is HERV-K10 (Ono, M. et al., 1986, J. Virol. 60:589-598). The sequences of the env gene of MMTV (Majors, I.E. and Varmus, H.E., 1983, J Virol 47:495-504) were aligned with sequences of the env gene of the human endogenous retrovirus HERV-K10 (Ono, M. et al., 1986, J. Virol. 60:589-598), using the IBI/Pustell Sequence Analysis Program. A region of 660 bp of low homology (16%) was localized between MMTV env gene sequences 976 and 1640 (Majors, I.E. and Varmus, H.E., 1983, J Virol 47:495-504). This

internal domain of the outer membrane of the env gene has only one glycosylation site and is highly conserved between strains. Two primers comprising 15 bp sequences at positions 976-990 (primer 1) and 1626-1640 (primer 3) were first synthesized. Later longer primers were synthesized (1N and 3N). An 18-mer sequence in the middle of the 660 bp MMTV env region (1388-1405) (primer 2) was used as a probe to identify the 660 bp sequence. A second oligomer probe was synthesized comprising the sequence 1554 to 1568 (primer 2a) to be used for hybridization when a sequence of around 250 bp (between positions 1388 and 1640) was amplified. For nested PCR reactions (Mullis, K.B. and Faloona, F.A., 1987, Meth Enzymol 155:335-350), another primer comprising sequences 1647 to 1661 (primer 4) was synthesized to be used with primer 1 in the first reaction and primers 2 and 3 in the second. Modified primers with GC clamps and extra sequences were also synthesized and used in the PCR (primers 1a and 3a). Another set of primers comprising sequences 974 to 1003 (5L) and 1558 to 1577 (3L) were subsequently developed because their T<sub>m</sub>'s matched and provided better amplification than the original primers. The sequences are represented in Table 1. All of them were productive in amplification reactions.

**Table 1. Primer and probe sequences and location in mouse mammary tumor virus env gene**

	Designation	Sequence (5'-3')	Location
5	1	CCTCACTGCCAGATC	976-990
	1a	GGGAATTCCTCACTGCCAGATC	976-990
	1N	CCTCACTGCCAGATCGCCT	976-993
	2	TACATCTGCCTGTGTTAC	1388-1405
10	2N	CCTACATCTGCCTGTGTTAC	1386-1405
	2a	CCGCCATACGTGCTG	1554-1568
	3	ATCTGTGGCATACT	1640-1626
	3a	GGGAATTCATCTGTGGCATACT	1640-1626
	3N	ATCTGTGGCATACTAAAGG	1640-1621
15	4	GAATCGCTTGGCTCG	1661-1647
	5L	CCAGATCGCCTTTAAGAAGG	984-1003
	3L	TACAGGTAGCAGCACGTATG	1558-1577

#### 6.2.2. Detection of MMTV-Like Env Gene Sequences in Human Breast Tumor DNA

20 PCR was performed on DNA extracted from breast cancer tissues, normal breast tissues and from the plasmid containing the env gene of MMTV, using primers 1 and 3. Photographs of the ethidium bromide stained gels of the PCR product reveal the presence of an

25 approximately 660 bp sequence in some of the tumors, (Fig. 1A, lanes 1 and 3) but not in the normal tissue samples (Fig. 1A, lanes 2 and 4). As a positive control the MMTV env gene was also amplified (Fig. 1A, lane E). Similar results were obtained with modified

30 primers 1a, 3a, 3L and 5L. Southern blot hybridization of the gel with <sup>32</sup>P-labeled 18-mer oligonucleotide (primer 2) indicated that this internal sequence was present in the amplified material (Fig. 1B) and that the bands in the gel were not artifactual.

35 Our initial effort was to analyze a representative sample of breast cancer specimens as well as normal

tissues and other tumors. To date 343 breast tumors have been processed, DNA extracted and PCR performed. Of these 343 tumors, 314 were carcinomas and 29 were fibroadenomas. Amplification of sequences of 660 bp  
5 was observed in 121 of the carcinomas (38.5%) and in 2 of the 29 fibroadenomas (6.9%). These sequences were confirmed to be MMTV env gene-like sequences by hybridization with the labeled specific probe containing the internal sequences. These sequences  
10 were not detected in the DNAs extracted from 20 normal organs, 23 cancers from other organs and 26 samples of blood lymphocytes including 7 from breast cancer patients whose breast specimens were positive. From 107 samples of normal breast obtained from reduction  
15 mammoplasties, 2 were positive (1.8%). In addition to DNA from lymphocytes from seven positive patients, DNA from their normal breast tissue of the operated breast was tested in 4 cases. All were negative (Table 2). Finally, DNA of the MCF-7, and ED (a cell line  
20 developed in our laboratory from the pleural effusion of a patient with an env -positive breast tumor) breast cancer cell lines were shown to contain the 660 bp MMTV env gene-like sequences (Table 3), while four other breast cancer cell lines were positive only for the  
25 250 bp sequence (T47-D, BT-474, BT-20 and MDA-MB-231).

**Table 2. Detection of MMTV env gene-like sequences in human DNA extracted from fresh or frozen tissues**

5	Sample	Number	MMTV <u>env</u> gene sequences	% Positive
	Breast Carcinomas	314	121	38.5%
	Breast Fibroadenomas	29	2	6.9%
10	Normal Breasts	107	2	1.8%
	*Normal Breasts	4	negative	
	Tumors other than breast	23	negative	
	Normal tissues	20	negative	
15	Lymphocytes	26	negative	
	**Lymphocytes	7	negative	
	* Histologically normal tissue from same breast as positive cancer.			
20	** Lymphocytes from breast cancer patients who were positive for MMTV <u>env</u> gene sequences in the tumor.			

**Table 3. Detection of MMTV env gene-like sequences in DNA from human cell lines in culture**

---

	Human Cell Lines		MMTV <u>env</u> gene sequence
5	MC-7	(breast carcinoma)	positive
	T47-D	" "	negative
	BT-20	" "	negative
	MDA-MB-231	" "	negative
	ZR-75-1	" "	negative
10	SK-BR 3	" "	negative
	BT474	" "	negative
	ED	" "	positive
	MCF-10	(normal breast)	negative
	HB-447	" "	negative
15	HL-60	(promyelocytic leukemia)	negative
	K562	(erythroleukemia)	negative
	Jurkat	(T cell leukemia)	negative
	Hep 6-2	(hepatoma)	negative

The nested polymerase reaction was used in several instances to increase sensitivity and specificity, thus reducing the probability of false positives. In Fig. 2, results of a representative nested reaction are shown using primers 1 and 4 in the first reaction (Fig. 2A) and 2 and 3 for the 2nd reaction. The specificity of the reaction can be seen in the 2nd amplification (Fig. 2B).

To study a large number of samples and to be able to perform archival studies, PCR of paraffin-embedded tissue sections was also carried out. Primers 2 and 3 were used to amplify a 250 bp sequence within the 660 bp stretch when DNA was extracted from paraffin-embedded tissue sections since larger size sequences are difficult to amplify after fixation. Tumor DNA was amplified (Fig. 3A, lanes 2-5) whereas normal breast DNA was not (Fig. 3A, lane 1). The identification of

this 250 bp sequence with the MMTV-like env gene was confirmed by hybridization with an internal probe (primer 2a) as shown in Fig. 3B. Using this procedure we have analyzed 151 breast cancer samples and found that 60 (39.7%) possess the 250 bp sequence. Of the 27 normal breast samples obtained from reduction mammoplasties assayed by this procedure, one was positive (3.7%). These results, in conjunction with those obtained from lymphocytes and from normal breast tissue of patients whose breast cancer was PCR positive, indicate that MMTV-like sequences are present in a significant number of human breast cancer DNA which cannot be explained by DNA polymorphism.

#### 6.2.3. Cloning and Sequencing of the MMTV-Like Env Gene Sequences

To find out whether there was homology to MMTV env gene throughout the whole 660 bp stretch, the product of the PCR from 8 different tumors was cloned and sequenced. In Fig. 4 the sequence of different clones comprising around 600 bp are represented, as aligned to the MMTV env gene sequence of the GR and BR6 strains (Redmon, S. and Dickson, C., 1983, EMBO J. 2:125-131). This domain of the env gene in the GR strain is 100% homologous to the C<sub>3</sub>H strain and 98% to the BR6 strain (Majors, I.E. and Varmus, H.E., 1983, J. Virol. 47:495-504; Moore, R. et al., 1987, J. Virol. 61:480-490). Evaluation of the clones indicated that homology to MMTV env gene varied from 95% to 99%. Another seven clones comprising only 250 bp were also sequenced. Homology to MMTV env gene varied from 95% to 99% (data not shown). When compared to the human endogenous provirus HERV-K10, the homology of all the clones was less than 15%. When compared against all known viral and human genes (more than 130,000 entries) using the 1B1 MacVector GenBank and GCG programs, the highest homology recorded was 18%.



#### 6.2.4. Southern Blot Analysis Using Cloned Sequences

To investigate whether the env gene-like sequences were present in human DNA, Southern blot hybridization was performed using the cloned sequence as probe. DNAs from normal breast tissues, env positive or negative breast tumors, tumors other than breast and breast cancer cell lines were restricted with EcoRI and in some instances with PstI, BglII or KpnI. EcoRI is a frequent cutter restriction enzyme that digests MMTV proviral DNA between env and pol genes. Four different cloned 660 bp sequences were used as probes after labeling with <sup>32</sup>P by random prime-labeling. Results of some of the Southern blot hybridization experiments are shown in Fig. 5. They reveal the presence of a labeled restriction fragment migrating at approximately 7-8 kb in breast cancer DNA, in ED and two fragments in MCF-7 cells. Different restriction patterns were observed with the other three enzymes. The 660 bp sequence was absent in 10 normal tissues, 10 fibroadenomas and 10 tumors from other tissues. It is important to emphasize that hybridization conditions for these experiments were stringent (as described in Section 6.1) to avoid interference with endogenous sequences that might interact with the probes.

### 7. Example: Detection of a Retrovirus Proviral Fragment in Human Breast Cancer Cells and Tissues

#### 7.1. Materials and Methods

To detect longer retrovirus proviral fragments in breast cancer samples, DNA was extracted from breast cancer carcinoma tissue samples as described above in Section 6.1. Two rounds of long PCR was performed on the DNA primers 5L (SEQ ID NO:11) and LTR3 (SEQ ID NO:19). The primer 5L contains nucleotides 7370-7395 of the MMTV BR6 genome (5'-3': CCAGATCGCCTTTAAGAAGG) (SEQ ID NO:11) and primer LTR3 contains nucleotides

9918-9927 of the MMTV BR6 genome (5'-3':  
CGAACAGACACAAAGCGACG) (SEQ ID NO:19). Long PCR was  
performed using protocols described by the manufacturer  
(Perkin Elmer, Foster City, CA). The amplified  
5 retroviral fragment isolated from the breast cancer  
sample was cloned into the TA cloning vector  
(Invitrogen) and automated sequencing was performed  
as described in Section 6.1.

## 7.2 Results

10 An approximately 2.6 kb retroviral fragment  
containing 1,228 bp of the 3' LTR sequence and 1,336 bp  
of the env gene sequence of a potential provirus was  
detected in a human breast carcinoma tissue sample by  
the long PCR technique using the 5L and LTR3 primers.  
15 The sequence of this retroviral fragment is shown in  
Fig. 9. (SEQ ID NO:20).

When compared with the two strains of MMTV C3H  
and BR6, the sequence homology was 90.8% and 90.7%,  
respectively, over the MMTV genomic fragment from  
20 nucleotides 7370-9937. When compared with the  
endogenous retroviral sequences (HUMERKA), sequence  
homology was only 58% in 36 bp and 71% in 74 bp.

## 8. Discussion

Search for virus-related sequences in human breast  
25 cancer has been hampered by great variation reported  
in previous studies, by the presence of endogenous  
retroviral sequences in human DNA and by the lack of  
sensitivity of the methods employed. The studies  
reported herein circumvent these deficiencies by  
30 focusing on sequences with low homology to human  
endogenous retroviruses, by investigating a large  
number of tumors and several types of controls and  
by using the most sensitive technology presently  
available.

35 The results indicate that unique MMTV env gene  
sequences were present in 38.5% of the breast cancer

samples analyzed and 39.7% of archival samples of breast cancer and that these sequences were absent in normal tissues including lymphocytes from patients with positive breast cancer and in cancers other than breast. Normal breast tissue and fibroadenomas had a low frequency (1.8 to 6.9%) of positive results. When cloned and sequenced, the sequences were found to be highly homologous to MMTV env gene, but not to the endogenous retroviral sequences. Furthermore, experiments in which the cloned amplified sequences were used for hybridization with DNA from breast cancer or normal tissues revealed that homologous DNA was only present in breast cancer DNA. The results also indicate that a human breast carcinoma sample contained an about 2.6 kb MMTV-like fragment comprised of 1,336 bp of the env gene and 1,228 bp of the 3' LTR.

The detection of MMTV env gene sequences in two fibroadenomas out of 29 and in two normal breast tissue samples out of 107 samples is of uncertain significance. Although such results could potentially be artifactual, and thus may represent false positives, they may alternatively indicate the presence of histologically unrecognized cells that were or will be neoplastic.

Ninety percent (90%) of the breast cancers tested were invasive ductal carcinomas, which reflects the prevalence of this type of neoplasm. Most patients were node-positive which is probably artifactual since it was necessary that tumor size be sufficiently large to provide an aliquot for research and tumor size correlates with node positivity.

It is unlikely that differences in homology between MMTV env gene and the cloned human sequences are generated by errors committed by the Taq polymerase. It has been estimated that the rate of nucleotide misincorporation is  $1 \times 10^{-5}$  per cycle (Ehrlich et al, 1991, Science 252:1643-1651) and

therefore, only a total of 0.32 nucleotides  
misincorporated should be expected in 660 bp after  
50 cycles. The differences in homology between clones  
from different patients is likely to represent  
5 heterogeneity of the env gene.

In contrast to earlier, ambiguous data associating  
MMTV-like sequences with human breast cancer, we have  
clearly demonstrated the existence of such sequences in  
breast cancer cells which cannot be explained by any  
10 known human endogenous retroviral sequence. Our data do  
not support the results of earlier studies which  
indicated that, as in the mouse, MMTV-like sequences  
were found in lymphocytes from two patients with breast  
cancer (Crepin, M. et al., 1984, Biochem. Biophys. Res.  
15 Comm. 118:324-331). The absence of MMTV env-like  
sequences in lymphocytes could reflect the fate of a  
unique lymphocyte subset over decades between initial  
encounter and the appearance of clinical breast cancer;  
alternatively, the human disease may differ from the  
20 mouse model. Results from attempts to identify unique  
MMTV-like pol gene sequences have shown that they  
cannot be distinguished from the reverse transcriptase  
sequences of endogenous retroviruses (Deen, K.C. and  
Sweet, R.W., 1986, J. Virol. 57:422-432).

25 The origin of the MMTV env gene-like and 3' LTR-  
like sequences found in tumor DNA could be the result  
of integrated MMTV-like sequences from a human mammary  
tumor virus. Polymorphism of endogenous retroviral  
sequences is conceivable but can be ruled out because  
30 these sequences were not detected in lymphocytes from  
the positive patients, in sections of the cancerous  
breast from which abnormal cells were absent, or in  
normal breast tissue from patients with MMTV env-like  
positive tumors. Recombination during tumorigenesis  
35 between endogenous sequences to resemble the MMTV env  
genes seems highly unlikely since no known gene or  
viral sequence is more than 18% homologous to the

660 bp sequence. The longer about 2.6 kb MMTV-like fragment detected in a human breast carcinoma had minimal homology (58% in 36 bp and 71% in 74 bp) to endogenous human retroviral sequences. Thus, the most conservative interpretation is that our findings represent exogenous sequences from an agent similar to MMTV. Recombination between endogenous and exogenous env gene sequences are known to accelerate the development of malignancies in mice (DiFronzo, N.L. and Holland, C.A., 1993, J. Virol. 67:3763-3770). Whether the MMTV-like sequences belong to an entire acquired provirus or to an exogenous fragment integrated into endogenous sequences, is presently not known. Experiments are in progress to distinguish between these possibilities.

Several genetic alterations have been identified in human breast cancer that can be useful as markers for prevention, detection or prognosis (reviewed in Runnenbaum, I. et al., 1991, Proc. Natl. Acad. Sci. USA 88:10657-10661). The BRCA1 and BRCA2 genes have recently been described. They account for at least 5% of breast cancer and are related to familial breast cancer (Miki, Y. et al., 1994, Science 266:66-71; Wooster, R. et al., 1994, Science 265:2088-2090). We have primary evidence that familial clustering of the MMTV env gene-like sequences occurs, accounting for an even higher percentage of cancers in affected families (Holland et al. 1994, Proc. Am. Assoc. Cancer Res 35:218). The presence of MMTV-like sequences may be correlated with special clinical disease status, may provide another potential molecular marker, and may distinguish a subset of human breast cancer for which viral etiology is tenable. This has implications for epidemiology, therapy and prevention.

Various publications are cited herein, the contents of which are hereby incorporated by reference in their entireties.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

(i) APPLICANT: HOLLAND, JAMES

5 (ii) TITLE OF THE INVENTION: DETECTION OF MAMMARY TUMOR VIRUS-LIKE  
SEQUENCES IN HUMAN BREAST CANCER

(iii) NUMBER OF SEQUENCES: 20

## (iv) CORRESPONDENCE ADDRESS:

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## 15 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette  
(B) COMPUTER: IBM Compatible  
(C) OPERATING SYSTEM: DOS  
(D) SOFTWARE: FastSEQ Version 1.5

## 20 (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: NOT YET ASSIGNED  
(B) FILING DATE: 08-NOV-1996  
(C) CLASSIFICATION:

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(A) APPLICATION NUMBER 08/555,394  
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## 35 (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 15 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

45 (v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCTCACTGCC AGATC

15

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGGAATTCCT CACTGCCAGA TC

22

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCTCACTGCC AGATCGCCT

19

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TACATCTGCC TGTGTTAC

18

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30

5 (ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:  
(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCTACATCTG CCTGTGTAC

20

(2) INFORMATION FOR SEQ ID NO:6:

10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 15 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
20 (vi) ORIGINAL SOURCE:  
(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCGCCATACG TGCTG

15

(2) INFORMATION FOR SEQ ID NO:7:

25 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 15 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:  
(ix) FEATURE:

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATCTGTGGCA TACCT

15

(2) INFORMATION FOR SEQ ID NO:8:

40 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 23 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:  
(ix) FEATURE:



(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGAATTCAT CTGTGGCATA CCT

23

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATCTGTGGCA TACCTAAAGG

20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAATCGCTTG GCTCG

15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCAGATCGCC TTTAAGAAGG

20

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TACAGGTAGC AGCACGTATG

20

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Leu Lys Arg Pro Gly Phe Gln Glu His Glu Met Ile  
1 5 10

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Gly Leu Pro His Leu Ile Asp Ile Glu Lys Arg Gly  
1 5 10

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE: N-terminal  
(vi) ORIGINAL SOURCE:

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Thr Asn Cys Leu Asp Ser Ser Ala Tyr Asp Thr Ala  
1 5 10

(2) INFORMATION FOR SEQ ID NO:16:

10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: peptide  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE: N-terminal  
(vi) ORIGINAL SOURCE:

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Asp Ile Gly Asp Glu Pro Trp Phe Asp Asp  
1 5 10

(2) INFORMATION FOR SEQ ID NO:17:

25 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 662 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

35 TCCTCACTGC CAGATCGCCT TTAAGAAGGA CGCCTTCTGG GAGGGAGACG AGTCTGCTCC 60  
TCCACGGTGG TTGCCTTGCG CCTTCCCTGA CCAAGGGGTG AGTTTTTCTC CAAAAGGGGC 120  
CCTTGGGTTA CTTTGGGATT TCTCCCTTCC CTCGCCTAGT GTAGATCAGT CAGATCAGAT 180  
TAAAAGCAAA AAGGATCTAT TTGGAAATTA TACTCCCCCA GTCAATAAAG AGGTTCATCG 240  
ATGGTATGAA GCAGGATGGG TAGAACCTAC ATGGTTCTGG GAAAATTCTC CTAAGGATCC 300  
40 CAATGATAGA GATTTTACTG CTCTAGTTCC CATAAGAAAT TGTTTCGCTT AGTTGCAGCC 360  
TCAAGATATC TTATTCTCAA AAGGCAGGAT TTCAGGAACA TGAGATGATT CCTACATCTC 420  
TGTGTTACTT ACCCTTATGT CATATTATTA GGATTACCTC AGCTAATAGA TATAGAGAAA 480  
GAGGATCTAC TTTTCATATT TCCTGTTCTT CTTGTAGATT GACTAATTGT TTAGATTCTT 540  
CTGCCTACGA CTATGCAGCG ATCATAGTCA AGAGGCCGCC ATACGTGCTG CTACCTGTAG 600  
45 ATATTGGTGA TGAACCATGG TTTGATGATT CTGCCATTCA AACCTTTAGG TATGCCACAG 660  
AT 662

(2) INFORMATION FOR SEQ ID NO:18:

50 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 663 base pairs  
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

5	TCCTCACTGN	CAGATCGCCT	TTAAGAAGGA	CGCCTTCTGG	GAGGGAGACG	AGTCTGCTCC	60
10	TCCACGGTGG	TTGACTTGCG	CCTTCCCTGA	CCAGGGGGTG	AGTTTTTCTC	CAAAAGGGGC	120
	CCTTGGGTTA	CTTTGGGATT	TCTCCCTTCC	CTCGCCTAGT	GTAGATCAGT	CAGATCAGAT	180
	TAAAAGCAAA	AAGGATCTAT	TTGGAATTA	TACTCCCCCT	GTCAATAAAG	AGGTTTCATCG	240
	ATGGTATGAA	GCAGGATGGG	TAGAACCTAC	ATGGTTCTGG	GAAAATTCTC	CTAAGGATCC	300
15	CAATGATAGA	GATTTTACTG	CTCTAGTTCC	CATACAGAAT	TGTTTCGCTT	AGTTGCAGCC	360
	TCAAGATATC	TTATTCACAA	AAGGCAGGAT	TTCAAGAACA	TGACATGAAT	CCCTACATCT	420
	CTGTGTTACT	TACCCTTATG	CCANANTATT	AGGATTACCT	CAGCTAATAG	ATATAGAGGA	480
	AGAGGATCTA	CTTTTCATAT	TTCTGTCTCT	TCTTGTAGAT	TGACTAATTG	TTTAGATTCT	540
	TCTGCCTACG	ACTATGCAGC	GATCATAGTC	AAGAGGCCGC	CATACGTGCT	GCTACCTGTA	600
20	GATATTGGTG	ATGAACCATG	GTTTGATGAN	NCTGCCANTC	AAACCTTTAG	GTATNCCACA	660
	GAT						663

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGAACAGACA CAAACACACG

20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 2598 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

	CGAACAGACA	CAAACACACG	AGAGGTGAAT	GTTAGGACTG	TTGCAAGTTT	ACTCAAAAAA	60
	CAGCACTCTT	TTATATCATG	GTTTACATAA	GCATTTACAT	AAGACTTGGA	TAAGTTCCAA	120
	AAGAACATAG	GAGAATAGAA	CACTCAGAGC	TTAGATCAAA	ACATTTGATA	CCAAACCAAG	180
5	TCAGGAAACC	ACTTGTCTCA	CATCCTTGTT	TTAAGAACAG	TTTGTGACCC	TGAACTTACT	240
	TAAACCTTGG	GAACCGCAAN	GTTGGGCTCA	TAAAGGTTAT	CCATTATAGC	TCATGCCAAA	300
	ATTATCTGCA	GAAATGTGTT	CCTAATTGTC	TAGCCACTGC	CCCCTCCCTT	GGTATAATGA	360
	AAATCTTTCC	CCCAACGTTT	ATCCCACTCC	CCTAGATAAA	TATAATCATG	TACCTGTTGT	420
	TTTATGTCGT	CTTTTCTTTC	CTGAGTTAAC	ACACACCAAG	GAGGTCTAGC	TCTGGCGAGT	480
10	CTTTCACGAA	AGGGGAGGGA	TCTGTACAAC	ACTTTATAGC	CGTTGACTGT	GACCCACCTA	540
	TCGAAATTTA	AATCGTATCT	TCCTGTATAT	GGTAGCGGGG	CGTCTGTTGG	TCTGTAGATG	600
	TAAGTCCCGG	TTGCCACCAC	CTGTCTCCTA	TTTTGACAAG	CGTACTCCTC	TTTCCCCTTT	660
	TTACTTCTAG	GCCTGAGGCC	CTTAGTCCTT	GCACCTGTTT	TTCAACTGAG	GTTGAGCGTC	720
	TCTTTCTATT	TTCTATTCCC	ATTTCTAACC	TTTGAATTTG	AGTAAATATA	GTGCTAAAAG	780
15	ACAAAGATTC	ATTTCTTAAC	ATCATGATTA	ATAATCGACC	TATTGGATTG	GTCTTATTGG	840
	TAAAAATATA	ATTTTTAGCA	AGCATTCTTA	TTTCTATTTT	TGAAGGACAA	AGTCGGTGTG	900
	GCTTGTAANA	GGAANTTGCC	TGTGGTCCTT	GCCCCACGAG	GAAGGTCGAG	TTCTCCGAAT	960
	TGTTTAGATT	GTAATCTTGC	ACAGAAGAGT	TATTAAGAAG	ATCAAGGGTG	AGAGCCCTGC	1020
	GAGCACGAAC	CGCAACTTCC	CCCAATAGCC	CCAGGCAAAG	CAGAGCTATG	CCAAGTTTGC	1080
20	AGCAGANAAT	GAGTATGTCT	TTGTCTGATG	GGCTCATCCG	CGTGCACGCA	GACGGGTCGT	1140
	CCTTGGTGGG	AAACAACCCC	TTGGCTGCTT	CTCTCCTAAG	TGTAGGACAC	TCTCGGGAGT	1200
	TCAACCATTT	CTGCTGCAGG	CGCGGCATTT	CCCCCTTTTT	TCTTTTTTAA	AAGAAGCACG	1260
	TTAAGATCTG	ACTGCACTTG	GTCAAGGCTC	TTCGCAAAGC	ACTGGAATAA	AACGGGGGAA	1320
	ATCATAAGTA	CTATGACCAA	AAGCAGGGCT	CCAACCTCCTA	TAAAAATGAA	ATATTGTGTT	1380
25	CTAATCCAAT	GGATTTAAAG	CCTTTACTCC	ATTGGCNAAG	GANTGANCCA	ACCCCTGAGG	1440
	TCCCTGCGTT	CAAATTTTTT	TGCTCNTATC	CTAATCCAAT	TGGTAACCCC	GTTTNTTTTT	1500
	GAAACTCATG	TCTTCAAATG	CCCAATAAAT	GAGCCCTGGT	TCTTTCCCAG	CTCTCAGAAG	1560
	CATTATACGG	NANAGGTGTG	ACACAGCATA	AAATCATAAT	TTGCATGACA	CCTAGTGGAC	1620
	ATTCTGGTCT	TTAAGTTTGC	CACATCTTGT	CCCAACTCTA	AAACTACTTC	TTCTAAAGCA	1680
30	TTAAGTCTAG	CTTTCAATTT	TAAGTCTATT	ATTCTTTGTT	CAGATNAGGC	TAATGTAACA	1740
	TTTCTATGAA	GATTATTAAC	AAACGTAGCA	GTTTGCATCT	CCTTAACTAA	GGCAGTAGTA	1800
	GCTACAGCAA	AGGAAGTGAT	AATAGCAATT	AAAGCAGATA	TGCCCAGAAT	AATGGCAGCG	1860
	ACGAATCGCT	TAGCTCGAAT	TAAATCTGTG	GCATACCTAA	AGGTTTGAAT	GGCAGAATCA	1920
	TCAAACCATG	GTTTCATCACC	AATATCTACA	GGTTACAACA	CATATGGCGG	CCCCTTGAAT	1980
35	ATGAATCGCT	GCATATCCGT	NGGCAAAAAA	TCTAACCATT	ATTCCTCCTN	CCNAAAAACG	2040
	GGATTTGAAA	NTTATNCCCC	TTNCCCCNAA	CCCANACCGA	GGTACCCCAT	AATGNNGGGG	2100
	GTATCTANAA	NAGGGCATAG	GGGTAAGAAA	AACGGCAGAG	NGGGATCNTT	TATGTTCTNGG	2160
	AAATTCTNGG	TTTGGGAGAA	TAAGATTCTG	GAGGCTGCAA	ATTAAGGGAA	ACATTNTGTA	2220
	TGGGGAATAG	AGCAGTAAAA	TCTCTATCAT	GGGGATCTTT	AGGGAGAATT	TTCCAGGAA	2280
40	CCAAGTAGGT	TCNAACCCAT	CNTGCTTCAT	ACCATCGATG	AACNTCTTTA	TTGACAGGGG	2340
	GAGTATAATT	TCCAAATAGA	TCCTTTTTGT	TTTTAATCTG	ATCTGACTGA	TCTACACTAG	2400
	GCGGGGGAAG	GGAGAAATCC	CAAAGTAACC	CAAGGGCCCC	TTTTGGAGAA	AAACTCACCC	2460
	CCTGGTCAGG	GAAGGCGCAA	GGCAACCACC	GTGGAGGAGC	AGACTCGTCT	CCCTCCCAGA	2520
	AGGCGTCCTT	CTTAAAGGCG	ATCTGGAGGA	GCAGACTCGT	CTCCCTCCCA	GAAGGCGTCC	2580
45	TTCTTAAAGG	CGATCTGG					2598

Claims

- 1     1.     A composition comprising an oligonucleotide primer  
2           which may be used to detect the presence of a  
3           nucleic acid molecule which (i) hybridizes to the  
4           env gene of a mouse mammary tumor virus; (ii) is  
5           present in at least 38 percent of DNA samples  
6           prepared from breast cancer tissue of different  
7           human subjects; and (iii) hybridizes to less than  
8           7 percent of DNA samples prepared from tissues  
9           other than breast cancer tissue from different  
10          human subjects.
- 1     2.     The composition of claim 1, wherein the  
2           oligonucleotide primer comprises the sequence  
3           CCTCACTGCCAGATC (SEQ ID NO:1).
- 1     3.     The composition of claim 1, wherein the  
2           oligonucleotide primer comprises the sequence  
3           GGGAATTCCTCACTGCCAGATC (SEQ ID NO:2).
- 1     4.     The composition of claim 1, wherein the  
2           oligonucleotide primer comprises the sequence  
3           CCTCACTGCCAGATCGCCT (SEQ ID NO:3).
- 1     5.     The composition of claim 1, wherein the  
2           oligonucleotide primer comprises the sequence  
3           TACATCTGCCTGTGTTAC (SEQ ID NO:4).
- 1     6.     The composition of claim 1, wherein the  
2           oligonucleotide primer comprises the sequence  
3           CCTACATCTGCCTGTGTTAC (SEQ ID NO:5).
- 1     7.     The composition of claim 1, wherein the  
2           oligonucleotide primer comprises the sequence  
3           CCGCCATACGTGCTG (SEQ ID NO:6).

- 1    8.    The composition of claim 1, wherein the  
2           oligonucleotide primer comprises the sequence  
3           ATCTGTGGCATACT (SEQ ID NO:7).
- 1    9.    The composition of claim 1, wherein the  
2           oligonucleotide primer comprises the sequence  
3           GGGAATTCATCTGTGGCATACT (SEQ ID NO:8).
- 1    10.   The composition of claim 1, wherein the  
2           oligonucleotide primer comprises a sequence  
3           selected from the group consisting of  
4           ATCTGTGGCATACTAAAGG (SEQ ID NO:9);  
5           GAATCGCTTGGCTCG (SEQ ID NO:10);  
6           CCAGATCGCCTTTAAGAAGG (SEQ ID NO:11); and  
7           TACAGGTAGCAGCACGTATG (SEQ ID NO:12).
- 1    11.   An essentially purified peptide encoded by a  
2           nucleic acid molecule which (i) hybridizes to  
3           a gene of MMTV; (ii) is present in at least  
4           20 percent of DNA samples prepared from breast  
5           cancer tissue of different human subjects; and  
6           (iii) is present in less than 5 percent of DNA  
7           samples prepared from tissues other than breast  
8           cancer tissue from different human subjects.
- 1    12.   An antibody which specifically binds to the  
2           peptide of claim 11.
- 1    13.   The peptide according to claim 11 which comprises  
2           the amino acid sequence LKRPGFQEHMI (SEQ ID  
3           NO:13).
- 1    14.   An antibody which specifically binds to the  
2           peptide of claim 13.
- 1    15.   The peptide according to claim 11 which comprises  
2           the amino acid sequence GLPHLIDIEKRG (SEQ ID NO:14).

- 1     16.    A method of diagnosing breast cancer in a human  
2            subject, comprising detecting the presence of a  
3            peptide encoded by a nucleic acid molecule which  
4            (i) hybridizes to the env gene of 3' LTR of a  
5            mouse mammary tumor virus; (ii) is present in at  
6            least 20 percent of DNA samples prepared from  
7            breast cancer tissue of different human subjects;  
8            and (iii) is present in less than 5 percent of DNA  
9            samples prepared from tissues other than breast  
10           cancer tissue from different human subjects.
- 1     17.    The method according to claim 16, wherein the  
2            presence of a peptide comprising the amino acid  
3            sequence LKRPGFQHEMI (SEQ ID NO:13) is detected  
4            by the binding of an antibody specific to the  
5            peptide.
- 1     18.    The method according to claim 16, wherein the  
2            presence of a peptide comprising the amino acid  
3            sequence GLPHLIDIEKRG (SEQ ID NO:14) is detected  
4            by the binding of an antibody specific to the  
5            peptide.
- 1     19.    The method according to claim 16, wherein the  
2            presence of a peptide comprising the amino acid  
3            sequence TNCLDSSAYDTA (SEQ ID NO:15) is detected  
4            by the binding of an antibody specific to the  
5            peptide.
- 1     20.    The method according to claim 16, wherein the  
2            presence of a peptide comprising the amino acid  
3            sequence DIGDEPWFDD (SEQ ID NO:16) is detected by  
4            the binding of an antibody specific to the  
5            peptide.
- 1     21.    A composition comprising an oligonucleotide primer  
2            which may be used to detect the presence of a



3        nucleic acid molecule which (i) hybridizes to a  
4        nucleic acid comprised of a sequence selected from  
5        the group consisting of the env gene and the 3'  
6        LTR of a mouse mammary tumor virus; (ii) is  
7        present in a substantial percentage of DNA samples  
8        prepared from breast cancer tissue of different  
9        human subjects; and (iii) hybridizes to less than  
10       5 percent of DNA samples prepared from tissues  
11       other than breast cancer tissue from different  
12       human subjects.

1    22.   The composition of claim 1, wherein the  
2        oligonucleotide primer comprises the sequence  
3        CCAGATCGCCTTTAAGAAGG (SEQ ID NO:11).

1    23.   The composition of claim 1, wherein the  
2        oligonucleotide primer comprises the sequence  
3        CGAACAGACACAAACACACG (SEQ ID NO:19).

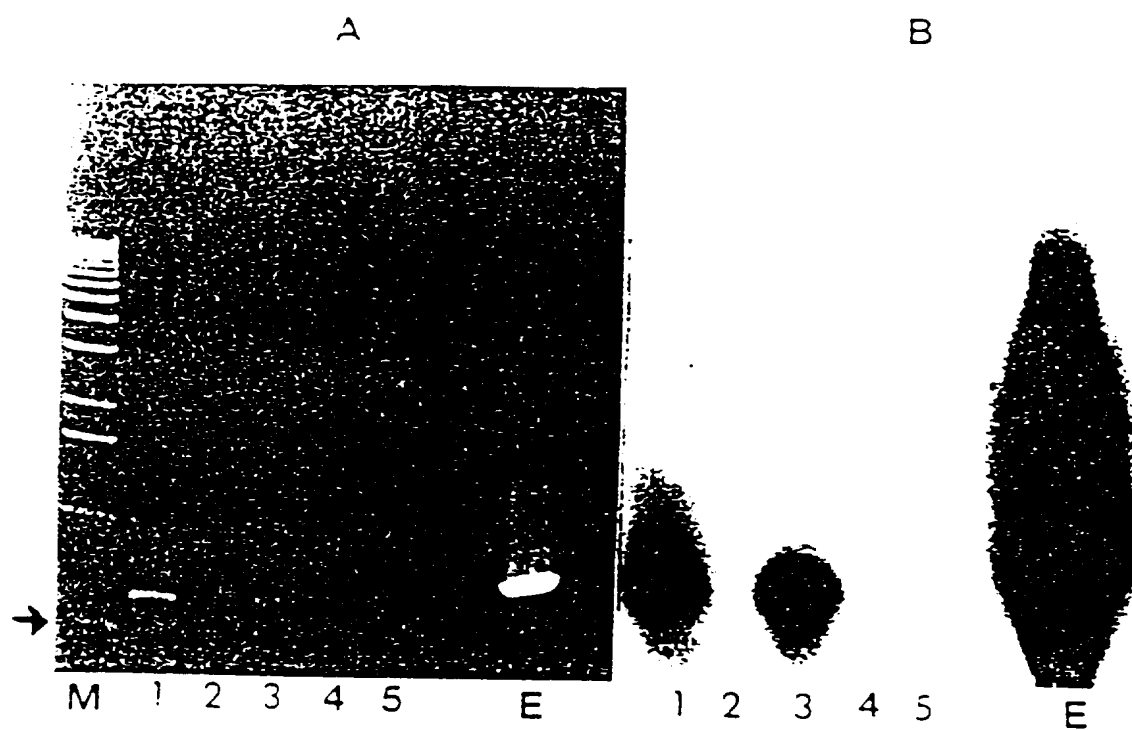


FIGURE 1

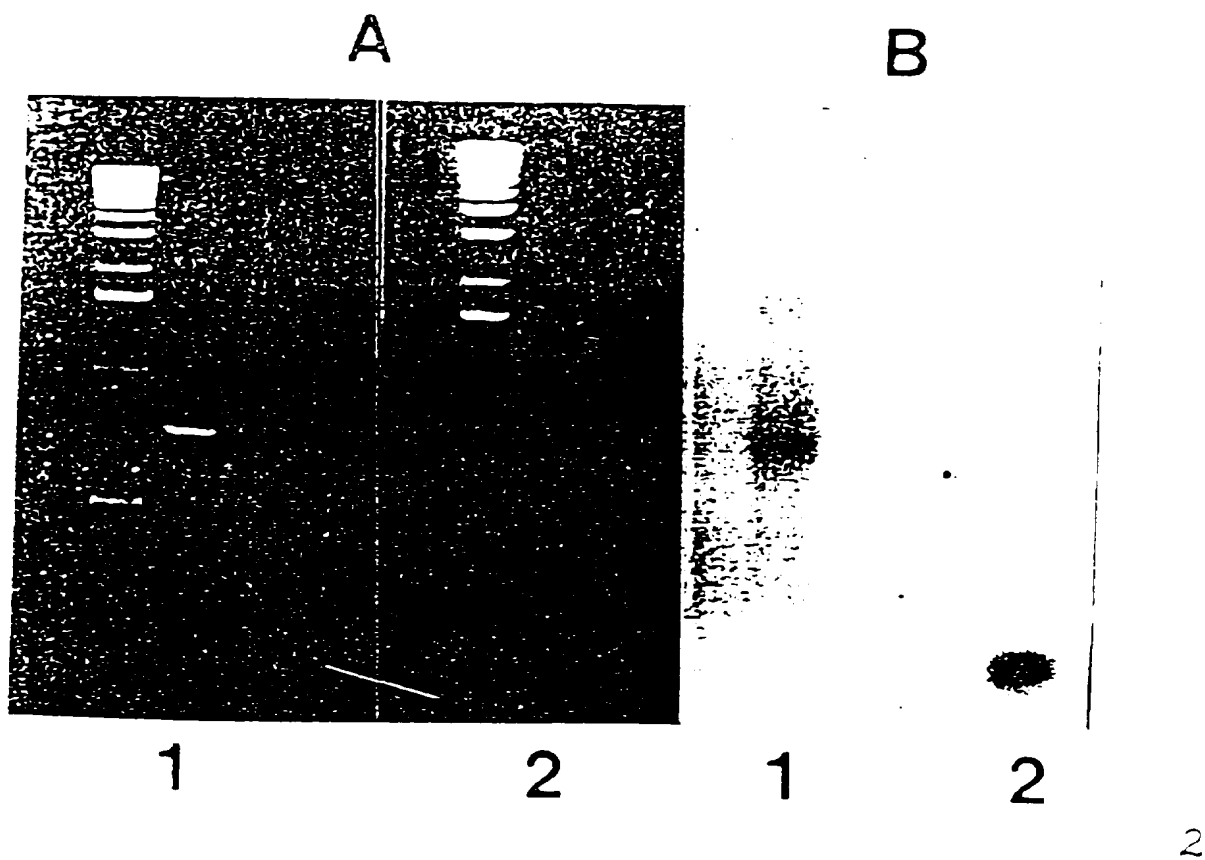


FIGURE 2

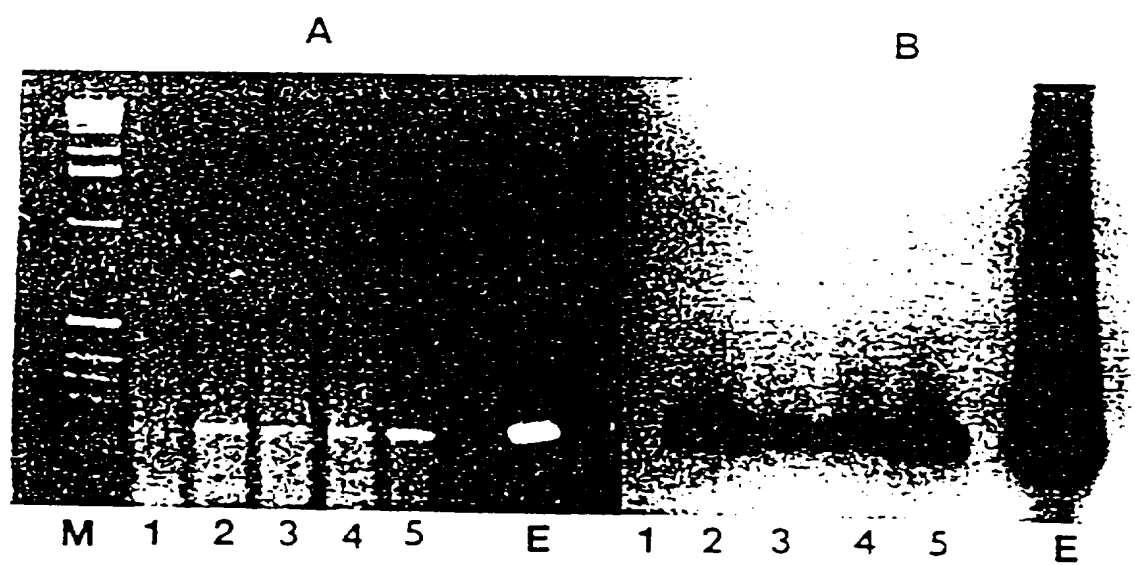


FIGURE 3

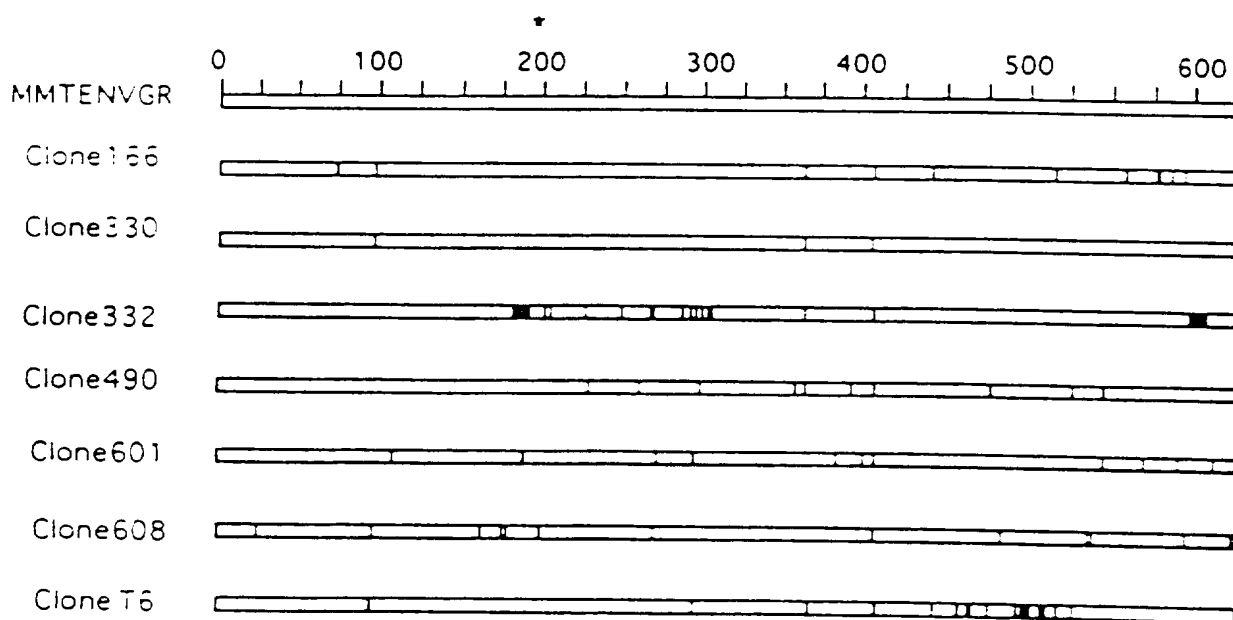
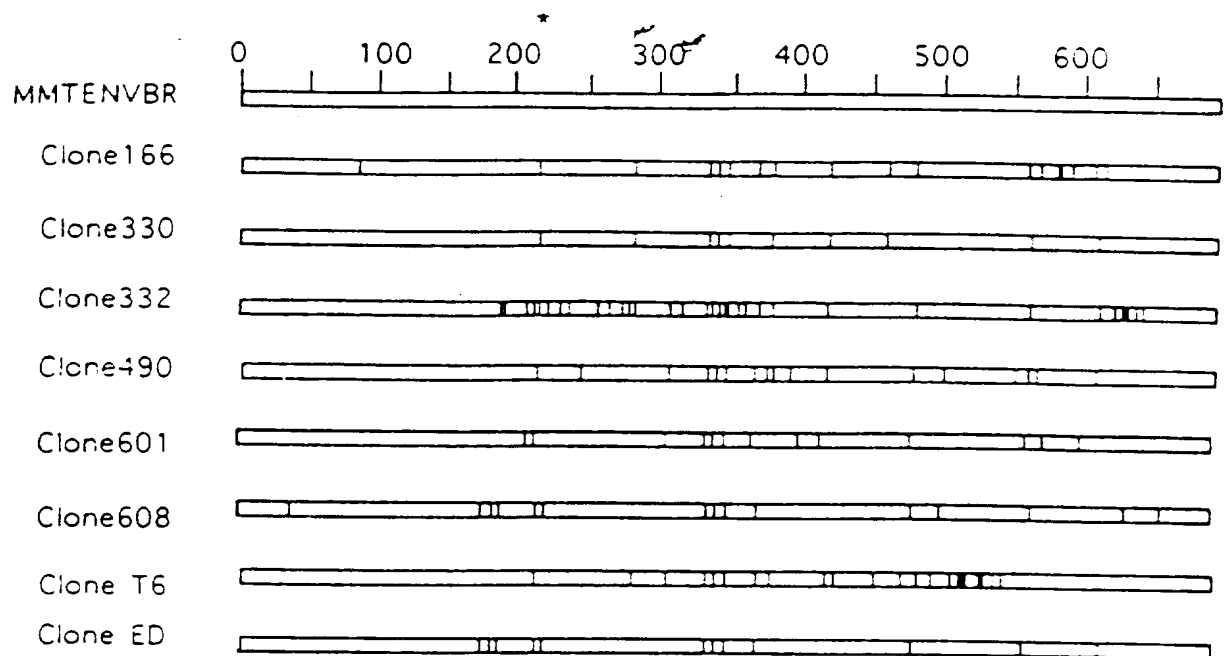


FIGURE 4

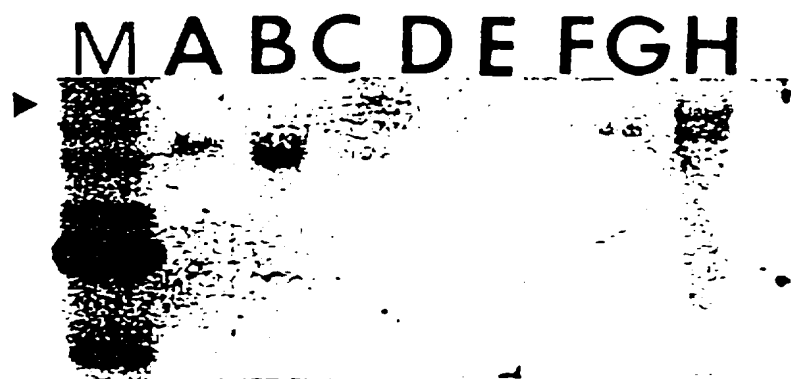


FIGURE 5

A B C D E F G H



FIGURE 6

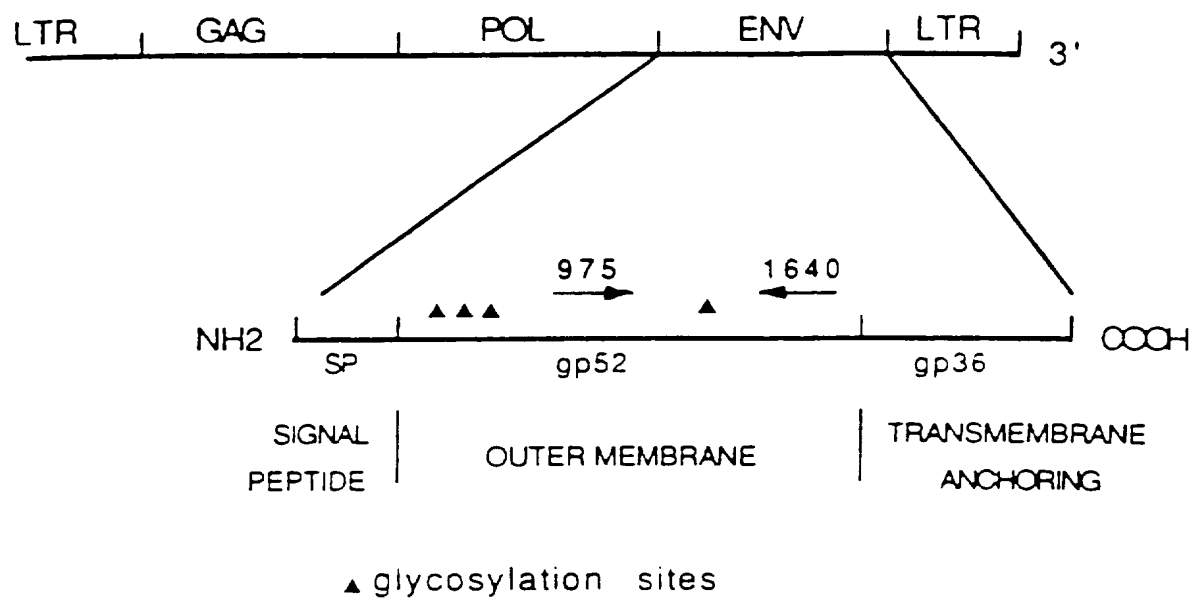


FIGURE 7



MMTENV  
( 1810 )  
MS1627.Seq

980 | 1000 | 1020 |  
TCTCACTGCGAGATGGCT TTAAGAAGGAGCGCTTCTGG GAGGAGACGAGTCTCTCTCC  
TCTCACTGCGAGATGGCT TTAAGAAGGAGCGCTTCTGG GAGGAGACGAGTCTCTCTCC  
5 10 15 20 25 30 35 40 45 50 55 60

MMTENV  
( 1810 )  
MS1627.Seq

1040 | 1060 | 1080 |  
TCCACGGTGGTTGGCTTGG CCTTCCTGACCAAGGGGTG ACTTTTCTCCAAAAGGGCC  
TCCACGGTGGTTGGCTTGG CCTTCCTGACCAAGGGGTG ACTTTTCTCCAAAAGGGCC  
65 70 75 80 85 90 95 100 105 110 115 120

MMTENV  
( 1810 )  
MS1627.Seq

1100 | 1120 | 1140 |  
CCTTGGGTACTTTGGGATT TCTCCCTTCCCTCCCTAGT GTAGATCACTCAGATCAGAT  
CCTTGGGTACTTTGGGATT TCTCCCTTCCCTCCCTAGT GTAGATCACTCAGATCAGAT  
125 130 135 140 145 150 155 160 165 170 175 180

MMTENV  
( 1810 )  
MS1627.Seq

1160 | 1180 | 1200 |  
TAAAGCAAAAAGGATCTAT TTGGAATTATACTCCCGCA GTCATAAAGAGGTTTCATCC  
TAAAGCAAAAAGGATCTAT TTGGAATTATACTCCCGCT GTCATAAAGAGGTTTCATCC  
185 190 195 200 205 210 215 220 225 230 235 240

MMTENV  
( 1810 )  
MS1627.Seq

1220 | 1240 | 1260 |  
ATGCTATGAAGCAGGATGGG TAGAACCTACATGGTTCTGG GAAAATTCCTTAAGGATCC  
ATGCTATGAAGCAGGATGGG TAGAACCTACATGGTTCTGG GAAAATTCCTTAAGGATCC  
245 250 255 260 265 270 275 280 285 290 295 300

MMTENV  
( 1810 )  
MS1627.Seq

1280 | 1300 | 1320 |  
CAATGATAGAGATTACTG CTCTAGTTCCCATACAGAA TGTTCCTTACTTTCACCC  
CAATGATAGAGATTACTG CTCTAGTTCCCATACAGAA TGTTCCTTACTTTCACCC  
305 310 315 320 325 330 335 340 345 350 355 360

MMTENV  
( 1810 )  
MS1627.Seq

1340 | 1360 | 1380 |  
TCAAGATATCTTATTCCAA AAGGCAGGATTTCAAGAAC TCAATG-ATCCTACATCT  
TCAAGATATCTTATTCCAA AAGGCAGGATTTCAAGAAC TCAATGATTCCTACATCT  
365 370 375 380 385 390 395 400 405 410 415 420

MMTENV  
( 1810 )  
MS1627.Seq

1400 | 1420 | 1440 |  
CTGTGTACTTACCTTATG CCACATATTAGGATTAAGT CAGCTAATAGATATAGAGA  
CTGTGTACTTACCTTATG CCACATATTAGGATTAAGT CAGCTAATAGATATAGAGA  
425 430 435 440 445 450 455 460 465 470 475 480

MMTENV  
( 1810 )  
MS1627.Seq

1460 | 1480 | 1500 |  
AGAAGATCTACTTTTCAAT TTCTGTCTCTCTTGTAGAT TGACTAATGTTTAGATTCT  
AGAAGATCTACTTTTCAAT TTCTGTCTCTCTTGTAGAT TGACTAATGTTTAGATTCT  
485 490 495 500 505 510 515 520 525 530 535 540

MMTENV  
( 1810 )  
MS1627.Seq

1520 | 1540 | 1560 |  
TCTGCTACGACTATGCAGC GATCATAGTCAAGAGGGGCG CATACGCTCTCTACCTGTA  
TCTGCTACGACTATGCAGC GATCATAGTCAAGAGGGGCG CATACGCTCTCTACCTGTA  
545 550 555 560 565 570 575 580 585 590 595 600

MMTENV  
( 1810 )  
MS1627.Seq

1580 | 1600 | 1620 | 1640 |  
GATATGCTGATGAACCATG GTTGTATGATCTGCAATC AAACCTTTAGGTATGCCACA GAT  
GATATGCTGATGAACCATG GTTGTATGATCTGCAATC AAACCTTTAGGTATGCCACA GAT  
605 610 615 620 625 630 635 640 645 650 655 660

FIGURE 8

CGAACAGACACACACACGAGAGGTGAATGTTAGGACCTTGCAAGTTTA  
CTCAAAAAACAGCACTCTTTTATATCATGGTTTACATAAGCATTACATAAGA  
CTTGGATAAGTTCCAAAAGAACATAGGAGAATAGAACACTCAGAGCTTAGAT  
CAAAACATTTGATACCAAACCAAGTCAGGAAACCACTTGTCTCACATCCTTG  
TTTTAAGAACAGTTTGTGACCCTGAACTTACTTAAACCTTGGGAACCGCAAN  
GTTGGGCTCATAAAGGTTATCCATTATAGCTCATGCCAAAATTATCTGCAGA  
AATGTGTTCCCTAATTGTCTAGCCACTGCCCCCTCCCTTGGTATAATGAAAAT  
CTTTCCCCCAACGTTTCATCCCCTAGATAAATATAATCATGTACCTGT  
TGTTTTATGTCGTCTTTTCTTCTGAGTTAACACACACCAAGGAGGTCTAGC  
TCTGGCGAGTCTTTCACGAAAGGGGAGGGATCTGTACAACACTTTATAGCC  
GTTGACTGTGACCCACCTATCGAAATTTAAATCGTATCTTCTGTATATGGTA  
GCGGGGCGTCTGTTGGTCTGTAGATGTAAGTCCCGGTTGCCACCACCTGTC  
TCCTATTTTGACAAGCGTACTCCTCTTTCCCTTTTACTTCTAGGCCTGAGG  
CCCTTAGTCTTGCACCTGTTCTTCAACTGAGGTTGAGCGTCTCTTTCTATTT  
TCTATTCCCATTTCTAACCTTTGAATTTGAGTAAATATAGTGCTAAAAGACAA  
AGATTCATTTCTTAACATCATGATTAAATAATCGACCTATTGGATTGGTCTTATT  
GGTAAAAATATAATTTTTAGCAAGCATTCTTATTTCTATTCTGAAGGACAAA  
GTCGGTGTGGCTTGTAANAGGAANTTGGCTGTGGTCTTGCCCCACGAGGA  
AGGTGCGAGTTCTCCGAATTGTTTAGATTGTAATCTTGACAGAAAGAGTTATTA  
AAAGAATCAAGGGTGAGAGCCCTGCGAGCACGAACCGCAACTTCCCCCAAT  
AGCCCCAGGCAAAGCAGAGCTATGCCAAGTTGCAGCAGANAATGAGTATG  
TCTTTGTCTGATGGGCTCATCCGCGTGCACGCAGACGGGTCTCCTTGGTG  
GGAAACAACCCCTTGGCTGCTTCTCTCCTAAGTGTAGGACACTCTCGGGAG  
TTCAACCATTCTGCTGCAGGCGCGGCATTTCCCTTTTCTTTTTTAAAA  
GAAGCACGTTAAGATCTGACTGCACCTGGTCAAGGCTCTTCGCAAAGCACT  
GGAAAATAACGGGGAAAATCATAAGTACTATGACCAAAGCAGGGCTCCAA  
CTCCTATAAAAATGAAATATTGTGTTCTAATCCAATGGATTTAAAGCCTTTAC  
TCCATTGGCNAAGGANTGANCCAACCCCTGAGGTCCCTGCGTTCAAATTTTT  
TTGCTCNTATCCTAATCCAATTGGTAACCCCGTTTNTTTTTGAACTCATGTC  
TTCAAATGCCCAATAAATGAGCCCTGGTTCTTTCCCAGCTCTCAGAAGCATT  
ATACGGNANAGGTGTGACACAGCATAAAATCATAATTTGCATGACACCTAGT  
GGACATTCTGGTCTTTAAGTTTGCCACATCTTGTCCCAACTCTAAACTACTT  
CTTCTAAAGCATTAAGTCTAGCTTTCAATTTTAAGTCTATTATTCTTTGTTGAG  
ATNAGGCTAATGTAACATTTCTATGAAGATTATTAACAAACGTAGCAGTTTGC  
ATCTCCTTAACCTAAGGCAGTAGTAGCTACAGCAAAGGAAGTGATAATAGCAA  
TTAAAGCAGATATGCCCAGAATAATGGCAGCGACGAATCGCTTAGCTCGAAT  
TAAATCTGTGGCATACTAAAGGTTTGAATGGCAGAATCATCAAACCATGGT  
TCATCACCAATATCTACAGGTTACAACACATATGGCGGCCCTTGAATATGA  
ATCGCTGCATATCCGTNGGCAAAAAATCTAACCATTATTCCTCCTNCCNAAA  
AACGGGATTTGAAANTTATNCCCCTTNCCCCNAACCCANACCGAGGTACCC  
CATAATGNNGGGGGGTATCTANAANAGGGCATAGGGGTAAGAAAAACGGCA  
GAGNNGGATCNTTTATGTTGNGGAAATTCNNGGTTTGGGAGAATAAGATTCT  
GGAGGCTGCAAATTAAGGGAAACATTNTGTATGGGGAATAGAGCAGTAAAA  
TCTCTATCATGGGGATCTTTAGGGAGAATTTTCCCAGGAACCAAGTAGGTTT  
NAACCCATCNTGCTTCATACCATCGATGAACNTCTTTATTGACAGGGGGAGT  
ATAATTTCCAAATAGATCCTTTTTGTTTTAATCTGATCTGACTGATCTACACT  
AGGCGGGGGGAAGGGAGAAATCCCAAAGTAACCCAAGGGCCCCCTTTTGGAG  
AAAAACTCACCCCTGGTCAGGGAAGGCGCAAGGCAACCCGTTGGAGGA  
GCAGACTCGTCTCCCTCCCAGAAGCGTCTTCTTAAAGGCGATCTGGAGG  
AGCAGACTCGTCTCCCTCCCAGAAGGCGTCTTCTTAAAGGCGATCTGG

FIGURE 9

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/17877

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : 435/6, 5, 91.2, 7.1, 7.2; 536 23.1, 24.3, 24.33; 530/388.1, 300

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 5, 91.2, 7.1, 7.2; 536 23.1, 24.3, 24.33; 530/388.1, 300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	REDMOND et al. Sequence and expression of the mouse mammary tumour virus env gene. The EMBO Journal. 1983, Volume 2, Number 1, pages 125-131. See entire document.	1-20
A	FAFF et al., Retrovirus-like particles from the human T47D cell lines are related to mouse mammary tumour virus and are of human endogenous origin. Journal of General Virology. 21 May 1992, Volume 73, pages 1087-1097. See abstract.	1-20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Z" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 04 FEBRUARY 1997	Date of mailing of the international search report 18 MAR 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DIANNE REES <i>JAB for</i> Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/17877

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CREPIN et al. Sequences Related to Mouse Mammary Tumor Virus Genome in Tumor Cells and Lymphocytes from Patients with Breast Cancer. Biochemical and Biophysical Research Communications. 13 January 1984, Volume 118, Number 1, pages 324-331. See entire document.	1-20
A	MESA-TEJADA et al. Detection in human breast carcinomas of an antigen immunologically related to a group-specific antigen of mouse mammary tumor virus. Proceedings of the National Academy of Sciences, USA. March 1978, Volume 75, Number 3, pages 1529-1533.	1-20*

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/17877

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C12Q 1/68, 1/70; C12P 19/34; C07H 21/02, 21/04; G01N 33/53; C07K 15/28; 5/00

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, BIOTECHABS, BIOTECHDS, CABA, CAPLUS, CANCERLIT, DGENE, DRUGU, EMBASES, MEDLINE, USPATFULL, TOXLIT, TOXLINE, JAPIO, WPIDS  
search terms: MMTV, mouse mammary tumor virus, PCR, hybridization, antibodies, immunoassays, Westerns, searched SEQ. ID. Nos.